Epidemiology, genetic diversity and clinical manifestations of arboviral diseases in Venezuela

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Summarizing Discussion

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In the last decades, arboviral diseases had an enormous impact on public health systems given the overwhelming increase of disease morbidity caused by these viruses in tropical and subtropical countries, especially in the Americas. The rapid expansion of various arboviruses and the explosive nature of their outbreaks have put on evidence: i) the potential of these vector-borne diseases to newly appear and rapidly expand (Weaver & Reisen, 2010; Hotez & Murray, 2017; CDC, 2019; WHO, 2019) as well as, ii) the difficulties to effectively control endemic and epidemic arboviral transmission. Within the arboviruses that show rapid expansion are dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) viruses. Dengue is the most relevant arboviral disease affecting annually an estimated 390 million people worldwide (Bhatt, 2013). In the Americas, a growing circulation of multiple DENV serotypes has shown implications for increased incidence of severe disease as a result of antibody-dependent enhancement (ADE; Katzelnick *et al*., 2017). Likewise, CHIKV and ZIKV have re-emerged in the last five years drawing special public attention due to the long-lasting sequelae produced by CHIKV (Schilte *et al*., 2013; Weaver 2015 *et al*., 2015; Elsinga *et al*., 2017; van Aalst *et al*., 2017; McHugh, 2018) and the link between ZIKV and microcephaly and other congenital disorders during pregnancy (Cauchemez *et al*., 2016; Calvet *et al*., 2016; Mlakar *et al*., 2016; de Paula Freitas *et al*., 2016).

Venezuela is endemic for dengue with the co-circulation of the four DENV serotypes. It is one of the countries with the highest proportion of severe cases in the Americas (PAHO, 2018). In addition, the epidemics of CHIKV in 2014 and ZIKV in 2016 swept the country causing a high burden of disease. These facts highlight the need for a better understanding of the epidemiological and molecular dynamics of these concurrent arboviral diseases. Our work provides a detailed epidemiological and spatial-temporal characterization of the introduction of a new arbovirus, CHIKV, into the country, coinciding with an outbreak of an endemic virus (DENV), stressing the need for accurate differential diagnosis. In addition, our work gives an update on the (molecular) epidemiology and evolution of DENV in Venezuela, revealing the frequency of circulating lineages and exploring their intra-host variability.

SPATIO-TEMPORAL PATTERN OF CHIKUNGUNYA AND A CONCURRENT DENGUE EPIDEMIC

The re-emergence of CHIKV followed a fast propagation pattern in the Americas during 2014 with more than 45 countries affected (Weaver & Forrester, 2015; Patterson *et al*., 2016). However, before this major epidemic took place in the Americas, CHIKV already showed its potential to cause major outbreaks in Kenya (2004), La Reunion Island (2005) and several islands in the Indian Ocean and India (Charrel *et al*., 2007; Pialoux *et al*., 2007; Gerardin *et al*., 2008; Sergon *et al*., 2008). In Venezuela, the 2014 chikungunya epidemic allowed us to describe the spatial and temporal dynamics of disease transmission during its first introduction into an immunological naïve population in the northern region of the country.

In Chapter 2, we were able to reconstruct a clear pattern of disease spread that followed a southwest spatial corridor with a maximum of traveled distance of 9.4 Km at a mean velocity of 82.9 m/day (at large scale). At local scale the epidemic showed spatio-temporal aggregation specially in the south of the capital city where lower socioeconomic status and crowded conditions are often found as a result of densely populated neighborhoods with deficiencies of public services. Such factors are known to increase the risk for DENV transmission and are associated to dengue clusters (hotspots) in urban areas of Venezuela (Barrera *et al*., 1995; Velasco *et al* 2014; Vincenti-Gonzalez *et al*., 2017) as well as in other countries (Phuong *et al*., 2008; Teixeira & Gonçalves, 2011; Steward-Ibarra *et al*., 2014). The strongest spatio-temporal clustering (75 significant clus-

ters) and higher relative risk occurred at 1-7 days and 25-150 m with an average speed of 69.9 ± 34 m/day within the clusters. These intervals/distances seem to be suitable for an increased likelihood of vector-host contact (viremic/non viremic) in our study population, as has been previously reported (Tran *et al*., 2004; Aldstadt, 2007; Aldstadt *et al*., 2012).

We found that while the whole epidemic unraveled within 28 weeks, the biggest spatio-temporal signal (peak timing) was detected around 70 days after the index case was reported in the west-central area of the capital city. The temporal dynamics of transmission (under the form of R0 and Rt) suggest high transmissibility in our population (R0 = 3.7; Rt = 4.5) and agree with previous estimates for CHIKV introductions into naïve populations (Boëlle *et al*., 2008; Yakob *et al*., 2013 and Perkins *et al*., 2015) and other *Aedes* mosquito-borne introductions (Chowell *et al*., 2002; Ferguson *et al*., 2016; Nishiura *et al*., 2016 and Johansson *et al*., 2011).

Furthermore, the epidemic of CHIKV took place during a concurrent DENV outbreak. Under this occurrence, the competition among viruses for the same ecologic niche (vector and host) was a determinant factor. In principle, the lack of herd immunity of the host population to CHIKV and a variable immune response to DENV made it favorable for CHIKV to spread in a very short period. The concomitant transmission of the viruses rose the questions on how each epidemic developed and what level of overlap occurred either at space, time or in their clinical/epidemiologic characteristics. Therefore, we further characterized the chikungunya outbreak of 2014 in the context of a concomitant epidemic of dengue in Carabobo state in Northern Venezuela (Chapter 3).

Firstly, by exploring the temporal distribution of reported cases of chikungunya and dengue, we found a delay on the official notification of chikungunya cases as well as underreporting of cases. Both events could have favored the further dispersion of CHIKV resulting from a belated response by the National Surveillance and Control Program. Our study provided evidence of high and significant temporal overlap in the transmission period of the two viruses and a similar timing of the epidemic peak (around EW 34). The concurrent occurrence of cases and similar epidemic peaks could be expected since both arboviruses share the same vector (Stapleford *et al*., 2016; Shiferaw *et al*., 2015) which in turn displays a seasonal occurrence that is also strongly influenced by climatic variables (Lee *et al*., 2016; Vincenti-Gonzalez *et al*., 2018). A similar overlap between the temporal pattern of arboviral transmission has been described before for DENV, CHIKV and ZIKV (Bisanzio *et al*., 2018).

All age groups were equally affected by CHIKV in agreement with lack of previous immunity, while higher prevalence of dengue was found in younger individuals and decreased with age (Thai *et al*., 2011; Velasco-Salas *et al.*, 2014). Although we expected a wider and more homogeneous spatial distribution of chikungunya cases within such a CHIKV immunologically-naive population, we found a spatial coherence between the distribution of chikungunya and dengue cases probably defined by mosquito seasonality and heterogeneity. This spatial distribution appeared to be aggregated in the central region of the state where the population density is higher and lower socioeconomic status and crowded living conditions are often encountered (Vincenti-Gonzalez *et al.*, 2017; Costa, *et al.*, 2018; Lizarazo *et al.*, 2019). The latter factors are considered as household risk factors associated with hotspots of transmission (Vincenti-Gonzalez *et al*., 2017). Altogether these factors in combination with the presence of the vector have proven to be relevant in dengue transmission under different urban settings including Venezuela and other countries (Honorio *et al*., 2009; Scandar *et al*., 2010; Teixeira *et al*., 2011; Sharma *et al*., 2014; Vincenti-Gonzalez *et al*., 2017).

Secondly, we focused on the clinical development and risks factors associated with the epidemic of chikungunya in Chapter 3. In our study, the most frequently reported symptoms by CHIKV-infected individuals were fever (98.9%), rash (93.4%), arthralgia (93.8%) and polyarthritis (90.9%). The latter symptoms are concordant with the characteristic symptoms of arthritis and arthralgia/joint pain present in >80% of patients linked to the Asian genotype of chikungunya during the outbreaks in the Caribbean and South America (Sahadeo *et al*., 2015; Mattar *et al*., 2015) and similar to the reported symptoms during the chikungunya epidemics of La Reunion and Italy (Rezza *et al*., 2007; Borgherini *et al*., 2007). Other likely chikungunya symptoms such as rash, headache and myalgia were reported in the range observed in La Reunion (Renault *et al*., 2007) and were considerably higher than in Colombia (Mattar *et al*., 2015). Interestingly, even though the introduction of CHIKV is linked to travelers returning from Dominican Republic, clinical symptoms (rash, headache, myalgia) present in our population were nearly absent in patients from Dominican Republic (Langsjoen *et al*., 2016). Furthermore, gastrointestinal signs and symptoms (nausea/vomiting, abdominal pain, and hepatomegaly) were less prevalent in our population than those reported in La Reunion (Geradin *et al*., 2008) but higher than the frequency of symptoms reported in Singapore by an Asian strain (Win *et al*., 2010; Lee *et al*., 2012). Importantly, the overlap in clinical manifestations with dengue was substantial, as expected. However, CHIKV and DENV co-infections could also be considered to occur in some cases (Ratsitorahina *et al*., 2008; Chang *et al*., 2010), confounding the clinical presentation. Despite similarities, significant differences were found. Rash, arthralgia, myalgia and headache were more likely to appear in chikungunya than dengue cases.

In Chapters 2 and 3, we conclude that the main determinants of the high epidemiological impact of chikungunya on the Venezuelan population were: i) the notification delay of suspected chikungunya cases (up to three weeks), ii) the difficulties to perform proper differential diagnosis and lastly, iii) a suspected widespread vector population due to lack of mosquito control measures. These factors highlight the need to increase preparedness and awareness in the management and control of arboviral diseases. Delays in case notification and acknowledgment of the development of an epidemic result in wrong or late allocation of resources to control the disease spread and inadequate use of field workers for vector control. Moreover, the rise in the co-circulation of arboviral infections with remarkable similar clinical features requires the use of diagnostic methods in the laboratory that enable differential diagnosis and the detection of mixed arboviral infections.

NEXT GENERATION SEQUENCING, ARBOVIRUS AND CUSTOMIZABLE PIPELINES

During the last decade shotgun metagenomics has been particularly promising and effective for diagnosis and public health surveillance of febrile illnesses (Greninger, 2015). With this technology we are able to simultaneously detect viruses, bacteria, and parasites in clinical samples by detecting and identifying genomic data without the need of targeting any organism in particular. Shotgun metagenomics does not require (specific) primers for typing allowing an unbiased approach to take place and the identification and typing of uncommon or new variants that would be missed when a primer-based method, such as Sanger sequencing, is used (Christenbury *et al*., 2010; Baronti *et al*., 2015). Moreover, in the last years reductions in instrument costs and improved library workflows have made metagenomic sequencing suitable for clinical diagnostic laboratories (Schlaberg *et al*., 2017). Therefore, in Chapter 4 we applied this method for the molecular characterization of DENV directly from clinical samples (sera and plasma). Specifically, we used short-read sequencing from Illumina platforms (San Diego, CA, USA) because of its multiplex possibility and its higher sequencing accuracy compared to other technologies that

were available at the time. In contrast to others, we did not use any viral RNA enrichment or amplification procedures before sequencing (Greninger *et al*., 2015; Kafetzopoulou *et al*., 2018). In this way, we accelerated the sequencing procedure, which is key in a diagnostic setting, and we prevented possible PCR amplification biases that may result in not detecting low frequency variants present in the samples. The estimated costs and the time to result of the shotgun metagenomic approach were calculated to be €130-170/sample and < 3 days, respectively, when using available commercial kits. These values are comparable to those of Sanger sequencing (Tan *et al*., 2015). Despite the similarity in costs, shotgun metagenomics allowed sample multiplexing and was able to detect low frequency variants and co-infections (see below) as has been reported before (Nasheri *et al*., 2017). Moreover, shotgun metagenomics generates whole genome sequences allowing typing at the highest possible resolution which is important in epidemiological and virus evolution studies (Houldcroft *et al*., 2017). In addition, shotgun metagenomics showed high sensitivity and specificity (up to 100%) when compared to RT-PCR or RT-qPCR (Lanciotti *et al*., 1992; CDC, 2017; Santiago *et al*., 2018) and it was able to detect DENV in clinical samples with as low as 5 viral copies/µL (Chapter 4). Therefore, shotgun metagenomics could be more cost-effective in a diagnostic/surveillance setting for DENV than currently used methods. However, some considerations have to be made when applying shotgun metagenomics, particularly the fact that when using direct patient's material, a high proportion of generated data (up to 90%) is actually human background and therefore not useful for pathogen genome reconstruction/analysis. On the other hand, the human reads may be interesting to record the host-response to the DENV infection. The quality of the sequencing data is highly dependent on the proper selection of reagents and tools used during the whole procedure, i.e., from sample collection to final result. For instance, in our case the input DNA quality had no effect on the sequenced data when using the TruSeq library preparation and sequencing reagents, but had a significant effect when using Nextera XT reagents for it, as previously reported (Tyler *et al*., 2016). This should be considered when implementing the method in clinical microbiology or public health laboratories.

We showed that shotgun metagenomics was able to detect multiple DENV serotypes in a single sample without targeting any specific serotype, despite the closely relatedness of DENV. The latter surmounts challenges like template concentration, sequence diversity, primer specificity and PCR amplification efficiency. Such challenges have been reported in previous efforts by Sanger or amplification-based Next Generation Sequencing (NGS) approaches (Christenbury *et al*., 2010; Baronti *et al*., 2015). The ability to detect multiple DENV serotypes together with the high throughput of the NGS platforms could facilitate the in-depth analysis of co-viral infections and their possible clinical manifestations. However, a metagenomic approach has been shown to be useful to detect other viral co-infections such as ZIKV and CHIKV as well (Sardi *et al*., 2016). Thus, considering the high prevalence of various arboviruses in some countries, the advantage of an unbiased method for detecting (arbo)viruses for diagnostic/surveillance purposes may be clear. High-throughput sequencing approaches also allow to study the interaction of inter- and intra-host virus variants (Nasheri *et al*., 2017). The latter are especially relevant during the course of the virus infection since it is known that during RNA virus replication, variants are generated also known as quasispecies (Holland *et al*., 1982; Holland *et al*., 1992; Eigen 1993, Vignuzzi *et al*., 2006). Such variants may have benefits (at intra-host population level), as they may increase the viral diversity. In this sense, viral populations arising during host infection constitute a cloud of genetically-linked mutants, rather than a homogeneous population (Poirier & Vignuzzi, 2017). It is not clear whether or not these variants under specific circumstances, allow the virus (quasispecies) to more easily and faster adapt to new environments and challenges encountered during infection (Eigen 1993, Vignuzzi *et al*., 2006). Shotgun metagenomics allowed

us to monitor such mutations in an unbiased manner. We detected mutations as single nucleotide variants (SNVs) in 71% of our samples. Some SNVs detected in DENV-1 and other serotypes represented multiple deleterious mutations such as frame shifts, intragenic stop-codons, nucleotide insertions or deletions that could affect viral pathogenesis by generating defective (interfering) viral particles (Pfeiffer & Kirkegaard, 2005; Choudhury *et al*., 2015). Interestingly, deleterious mutations were reported to be transmitted together with wild-type viruses of DENV-1 in Myanmar (Askov, 2006). It has been proposed that such deleterious mutations can serve as defective interfering particles (DIPs). DIPs interfere with virus replication and are non-replicative per se but are likely produced upon infection by any virus in vitro and in nature. DIPs could serve as decoys for the immune response of the host or could cause attenuation of disease severity, increasing the spread of the virus by allowing greater mobility of human hosts (Askov, 2006).

The sequenced viruses (Chapters 4 & 5) studied in this thesis cluster within distinct subpopulations of DENV, which could be related to the extensive DENV genetic variability in Venezuela or may be the result of multiple introductions of different subpopulations in the country as has been reported earlier (Rodriguez-Roche *et al.*, 2012; Ramirez *et al*., 2010). This is more extensively discussed below.

Obviously, the use of NGS in general, and shotgun metagenomics in particular, also faces some challenges. These include: i) the need of bioinformatic knowledge, ii) the lack of tools and computing power to analyze the amount of data obtained during the sequencing, iii) the need of storage of the "big data" generated, and iv) the need to reduce the time to get results. Therefore, in Chapter 6 we developed DEN-IM, a one-stop, user-friendly, freely available, containerized and reproducible workflow for the analysis of DENV sequencing data, both from shotgun and targeted metagenomics approaches. DEN-IM was designed to perform a comprehensive analysis without the requirement of extensive bioinformatics expertise in order to generate either assemblies or consensus of full DENV CDSs as well as to identify the serotype and genotype of the DENV present in the sample to further classify them in a phylogenetic tree diagram.

We decided to develop this workflow for identification and typing of DENV because despite the high burden of dengue, there are few tools available for these purposes. While some tools are available for viral read identification and assembly, such as VIP (Li *et al*., 2016), virusTAP (Yamashita *et al*., 2016) and drVM (Lin & Liao, 2017), none of them perform genotyping of the identified reads. Furthermore, there are initiatives focusing on the identification of the DENV serotype and genotype from NGS data. An example is The Genome Detective project (https://www.genomedetective.com/), that offers an online Dengue Typing Tool (https://www.genomedetective.com/app/typingtool/dengue/) relying on BLAST and phylogenetic methods in order to identify the closest serotype and genotype, but it requires as input assembled genomes in FASTA format. The same project also offers the Genome Detective Typing Tool (https://www.genomedetective.com/app/typingtool/virus/) (Fonseca *et al*., 2019) that identifies viruses present in a sample; however, DENV typing is lacking in this tool. Importantly, the Dengue Typing Tool is only available via an internet connection. In contrast, DEN-IM was developed in Nextflow as a stand-alone tool. It runs on any UNIX-like system and provides out-of-the-box support for several job schedulers (e.g., PBS, SGE, SLURM) and integration with containerized software like Docker or Singularity. While it has been developed to be ready-to-use for non-experts, not requiring any software installation or parameter tuning, it can easily be customized through the configuration files.

By using DEN-IM, we successfully analyzed two DENV datasets. The first comprised 25 shotgun metagenomic sequencing samples of variable serotype and genotype, including an in vitro spiked sample containing the four known serotypes. The second dataset consisted of 106 targeted metagenomic sequences of DENV 3 genotype III where DEN-IM allowed detection of the intra-genotype diversity. Thus, the advantage of DEN-IM resides on its two-pronged approach that combines assemblers and mapping in such way that all reads mapping DENV are retrieved before the assembly process, warranting better results. In some cases, the assembly process failed for the targeted metagenomics data, which may be related to errors introduced during the amplification process and resulting in low quality read ends. These are subsequently trimmed by the quality control block, potentially affecting the assembly process as the size and number of overlapping regions are diminished. DEN-IM's specificity was demonstrated by analyzing a dataset containing arboviruses other than DENV that did not result in false positive results.

EVOLUTIONARY HISTORY AND POPULATION DYNAMICS OF DENGUE VIRUSES IN VENEZUELA

Dengue in Venezuela has become a leading cause of morbidity that imposes a high burden in the national health system. This is particularly relevant in Aragua, which is a hyperendemic region for dengue with the co-circulation of all DENV serotypes and with cyclic epidemic periods. Dengue incidence in Aragua mimics closely the national incidence trend (Uzcategui *et al.*, 2001). This region faces dengue epidemics every 3-4 years (Vincenti-Gonzalez *et al.*, 2018).

In Venezuela, dengue was limited in the past to hypo-endemic epidemics with the circulation of a single serotype until 1989 (Barrera *et al.*, 2002) when the sequential introduction of serotypes changed the epidemiological landscape (Figure 1). In the year 2000, serotype 3 was re-introduced, causing a major epidemic, being this serotype the most prevalent during at least three continuous years. From there on, Venezuela was acknowledged as a hyperendemic country with co-circulation of all serotypes and an increasing occurrence of dengue hemorrhagic fever and severe dengue cases (SD), and with high rates among infants (Ramos-Castañeda *et al.*, 2017).

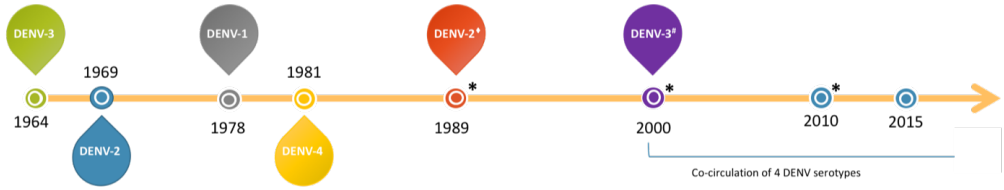

Figure 1. Sequential introduction of DENV serotypes in Venezuela since 1964. *Major dengue epidemics.

Since then, DENV epidemics of different magnitudes have occurred to date, with six major epidemics occurring in Aragua and in the rest of Venezuela within a period of fifteen years (2001, 2007, 2009–2010, 2012–2013, 2014 and 2015). After the re-emergence of DENV-3 in 1999, the distribution of serotypes has also changed through time with a dramatic shift in the proportions of the different DENV serotypes during the epidemics.

In Chapter 7, we investigated the evolutionary history and population structure of DENV in Venezuela, focusing on the mechanisms driving DENV evolution. Furthermore, we correlated the genetic diversity and population dynamics of DENV with the consecutive epidemics that occurred in Venezuela. Additionally, we employed whole-genome shotgun metagenomics as an unbiased high-throughput sequencing method to profile intra-host viral diversity across the entire coding

region of the four DENV serotypes.

Phylogenetic analyses from the genome sequences obtained in this thesis alongside the available genomes deposited in NCBI database showed a high genetic diversity of DENV serotypes in Venezuela despite of the fact that only a single genotype per serotype was detected. This high genetic variability among DENV serotypes has been attributed to multiple introductions of the same genotype from neighboring countries such as Colombia and Brazil (Weaver *et al*., 2009; Rodriguez-Roche *et al*., 2012). We did not find events of recombination in our analysis. However, DENV-1 showed a low temporal correlation of isolates in some phylogenetic clades, indicating temporal mixing that deviated from the root-to-tip analysis. Interestingly, the co-circulation of serotypes, genotypes or lineages generated a complex competition dynamic that affected the genetic diversity of serotypes. This was evident in the demographic reconstructed histories for all serotypes. The latter showed increases or decreases in the effective population size (Ne) or genetic diversity depending on the serotype. This genetic diversity is consistent with the rise in incidence and the dynamic of circulation of DENV-1, DENV-3 and DENV-4 in Aragua. Such correlation of events has been observed before after events of DENV introductions (Bennet *et al*., 2010; Cummings *et al*., 2004; Ooi *et al*., 2006). However, in the case of DENV-2, Ne remained constant throughout time. Thus, no correspondence with incidence was observed, which is similar to the genetic dynamics reported for the DENV-2 Asian/American genotype in the Americas (Wei & Li, 2017). Overall, our analysis indicates that DENV-3 serotype and its hyperendemicity had the highest impact on the landscape of genetic diversity of DENV in Aragua over a three years period, despite that all DENV serotypes co-circulated in the same region at the same time. Furthermore, the demographic reconstruction described a decay for DENV-1 and DENV-3 populations after periods of intense and prolonged transmission. The latter could indicate that herd immunity could play a role in such sustained decrease of diversity without a decrease in incidence.

Beyond this, the co-circulation of serotypes could also play an important role on disease clinical presentation as a result of consecutive heterologous infections. Indeed, it has been suggested that background immunity could play a role in clinical outcome. Specifically, a study indicates that DENV-1 serotype may be related to severe dengue cases in a secondary infection of DENV-2 and DENV-3 (Alvarez *et al*., 2006). Therefore, constant surveillance of the host immunologic status of the population and the viral genetic variability and the origin of such genetic diversity (in-situ evolution, introductions) should be considered in order to track possible evolutionary intermediates with epidemic potential.

CONCLUDING REMARKS

The gained insight into the spatial and temporal spread of an emergent arbovirus like CHIKV permits to establish routes of rapid spread for future arbovirus introduction permitting the generation of informed strategies for disease control and preparedness. Indeed, the chikungunya epidemic showed that transmission is likely to occur not only at a local scale within clusters driven by mosquito flying range and behavior (Rodhain *et al*., 1997; Getis *et al*., 2003; Harrington *et al*., 2005; Vazquez-Prokopec *et al*., 2010; Yoon *et al*., 2012) but also could happen by house-to-house human movement similarly to dengue (Stoddard *et al*., 2013). Hence, the long-distance spread of the chikungunya outbreak along major routes and motorways was likely due to human movement and passive dispersal of mosquitoes (e.g.: cars, cargo trucks) (Díaz-Nieto, 2016; Eritja *et al*., 2017) as the estimated speed of spread exceeded the scale of vector movements (Stoddard *et al*., 2009). Unravelling the individual movements in the course of a pathogen introduction as well as focal areas of transmission can help to model how a vector-borne pathogen may spread

through a population.

Additionally, in this thesis, we showed that shotgun metagenomics and bioinformatic tools can be employed for concomitant detection, identification and characterization of DENV serotypes/genotypes. The results confirm that metagenomics can be of value in clinical diagnostic settings and for surveillance, as has been reported before. (Syraka *et al*., 2010; Nasheri *et al*., 2017). Typically, for typing of DENV only some segments are sequenced (e.g. E/NS1, PrM/E); however, the high-throughput NGS assay applied in our study had more discriminatory power than methods that only target specific regions as it was able to generate full or near full length genomes. Moreover, the method allowed the detection of low frequency variants and co-infections with different DENV serotypes or other pathogens in a single reaction (Nasheri *et al*., 2017) making it more cost-effective in a diagnostic setting.

Lastly, we have presented an updated landscape of the molecular epidemiology of DENV in Venezuela, as well as the evolutionary processes that underlay the evolution of DENV serotypes in the country. In the latter case, purifying selection was found to be acting as a main force of selection, however we also found strong indication of positively selected sites in the polyprotein of DENV serotypes that indicates that some genetic changes are being fixed into the DENV viral population. We also showed that the introduction of DENV-3 serotype modified the genetic diversity landscape in Venezuela, thus positioning the competition among serotypes as an important factor in DENV lineage evolution in the country in addition to the alternating cycle of mosquito-human infection. Likewise, we showed the occurrence of high intra-host genetic diversity across the polyprotein of DENV serotypes and the presence of deleterious variants in the course of an infection. However, some questions remain such as those related to the relevance of the immunological background of the host and of secondary infections, and on how the host immune system shapes the variants during the course of an infection. Therefore, it is necessary to study the host-specific evolutionary paths to unravel how variants are fixed and consequently new lineages or genotypes variants are nurtured.

FUTURE PERSPECTIVES

The shotgun metagenomic results presented in this thesis indicate their great potential for detection and assembly of viral genomes even if present at low viral loads. Furthermore, the accuracy offered (99.9%) by the implemented Illumina platforms makes it ideal to capture within-sample diversity. However, in my opinion, and considering the current sequencing platforms, it would be of great interest to explore other sequencing technologies that can decrease the time to result or even could offer a real-time detection of pathogens. In particular, long read sequencing such as that one used in the Oxford Nanopore Technology (Third Generation sequencing) has two key advantages over short-read sequencing. First, the ability to perform real-time sequence analysis (Greninger *et al*., 2015) makes it suitable to be developed as a point-of-care test. Secondly, it's easiness to be deployed for outbreak investigation in field work. Lastly, the longer reads generated are also more suitable for characterizing viruses with segmented genomes in which re-assortments of segments occur (e.g. bunyaviruses) that cannot be revealed using short-read sequencing.

Importantly, better methods are required to follow up arboviral diseases not only on the clinical diagnostic laboratory but also in its natural environment (either urban or enzootic cycle). As we have shown in this thesis, arboviral diseases overlap in several aspects such as clinical presentation, vector, spatial distribution and seasonality. Yet, mechanisms to control disease spreading seem to work poorly or to be absent. It is not surprising that the general outlook worldwide

with regards to arboviral diseases seems to show a continued threat for the public health. This tendency is based on the global expansion of arboviruses which is heavily driven by the increase of globalization trend, human mobility, and fast vector dispersion (Kraemer *et al*., 2019), among other factors. For example, the recent and unexpected major epidemics (CHIKV, ZIKV) showed the need for active surveillance and tracking of arboviral diseases in order to detect them before reaching epidemic proportions.

Thus, in my opinion, the ultimate necessity in the clinical laboratory is a single-stop method that allows multiple pathogen detection and includes several layers of information that consents to keep track of circulating pathogens with increased genomic resolution (lineage/variant circulation). However, such information is only useful for fast and accurate detection of pathogens but it does not include information on the mobility/spread of the detected organisms. For this reason, additional information that is often collected alongside the samples for surveillance purposes needs to be explored in order to increase our knowledge on emergent and re-emergent diseases. An example of this is the hidden spatio-temporal dynamics of pathogen spread in the form of dates and geographic location in the widespread formularies for disease reporting. Indeed, we showed in Chapter 2 that it is possible to reconstruct the spread pattern of an arbovirus such as CHIKV. Therefore, a combination of both fast/accurate diagnosis and genomic tracing can help to keep track on: i) possible threats for urban settlements as has been recently shown for YFV surveillance in Brazil (Souza *et al*., 2019), and ii) evolution and spread of pathogens throughout different countries as shown for Zika in the Americas (Metsky *et al*., 2017). Together, the insights of the global spread of emergent arbovirus disease and a fast/accurate pathogen detection technique can aid to understand and predict/track epidemic waves of upcoming vector-borne infections.

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